MINIREVIEW

Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen

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Received 5 August 2008; revised 26 September 2008; accepted 28 September 2008.
First published online December 2008.

DOI:10.1111/j.1574-6941.2008.00633.x

Editor: Jim Prosser

Keywords
rumen; cellulose digestion; bacterial growth; model.

Abstract

Ruminant animals digest cellulose via a symbiotic relationship with ruminal microorganisms. Because feedstuffs only remain in the rumen for a short time, the rate of cellulose digestion must be very rapid. This speed is facilitated by rumination, a process that returns food to the mouth to be rechewed. By decreasing particle size, the cellulose surface area can be increased by up to 10^6-fold. The amount of cellulose digested is then a function of two competing rates, namely the digestion rate (K_d) and the rate of passage of solids from the rumen (K_p). Estimation of bacterial growth on cellulose is complicated by several factors: (1) energy must be expended for maintenance and growth of the cells, (2) only adherent cells are capable of degrading cellulose and (3) adherent cells can provide nonadherent cells with cellodextrins. Additionally, when ruminants are fed large amounts of cereal grain along with fiber, ruminal pH can decrease to a point where cellulolytic bacteria no longer grow. A dynamic model based on STELLA® software is presented. This model evaluates all of the major aspects of ruminal cellulose degradation: (1) ingestion, digestion and passage of feed particles, (2) maintenance and growth of cellulolytic bacteria and (3) pH effects.

Introduction

Humans have utilized microorganisms for more than 6000 years (Stanier *et al*., 1976). This utilization, first manifested in methods of food preservation, was gradually expanded to include alcoholic beverages and a variety of other fermentation products (e.g. solvents and antibiotics). However, an often-overlooked use of microorganisms was the domestication of ruminants. Humans and other simple-stomached animals cannot digest cellulose, the world's most abundant organic polymer, but ruminant animals provide a habitat (the rumen) for anaerobic microorganisms that can (Hungate, 1966). Domesticated ruminants allowed humans to expand their geographic range, develop more stable food supplies and create complex communities (Russell, 2002). Weimer (1992) estimated that the collective ruminal volume of domesticated cattle, sheep and goats (*c*. 2 billion animals) was nearly 100 billion liters, and concluded that, on a volume basis, the rumen is the 'world's largest commercial fermentation process.' The ability of ruminal fermentation to digest cellulose is typically highlighted as its primary attribute, but there is another significant nutritional aspect. Simple-stomached animals depend on feed for 10 essential amino acids, but ruminants can harvest microbial protein to meet this requirement. In ruminants, as much as 90% of the amino acids reaching the small intestine are derived from ruminal microorganisms; the efficiency of microbial growth is very important (Stern *et al*., 1994). However, it should be noted that while protozoal cellulose degradation may be low, protozoa are active consumers/degraders of both dietary starch and bacterial cells, and thus the amount of protozoal biomass in the rumen can approach that of the bacterial biomass, although most protozoal biomass does not escape the rumen (Weller & Pilgrim, 1974).

We have chosen to focus our attention on bacterial cellulose digestion and growth because the bacteria are essential. Some cellulose is also digested by protozoa, but *in vivo* and *in vitro* studies have indicated that their activity is much less than that of bacteria (Klopfenstein *et al*., 1966; Williams & Coleman, 1997; Lee *et al*., 2000). When very poor-quality forages are fed, fungi also play a role in cellulose digestion (Bauchop, 1979). However, ruminal

fungi are inhibited by bacterially produced, bacteriocin-like substances and are typically present when large amounts of cereal grain are fed (Dehority & Tirabasso, 2000). The purposes of this minireview are to (1) review our knowledge of ruminal cellulose digestion and factors affecting the production of bacterial cells, (2) provide a quantitative analysis of ruminal cellulose digestion and bacteria growth, (3) perform sensitivity analyses to assess the relative importance of physiological characteristics, (4) determine whether our current knowledge is adequate to explain *in vivo* cellulose digestion and its availability to the animal and (5) identify potential gaps in our understanding with an outlook for future research.

Ruminal cellulolytic bacteria

After developing methods for the cultivation of strictly anaerobic bacteria, Robert Hungate (1950) isolated predominant cellulolytic bacteria from the rumen and classified them as *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. Some strains of *Butyrivibrio fibrisolvens* are weakly cellulolytic, but it is doubtful that they play a significant role in ruminal cellulose degradation (Bryant & Small, 1956). Other species have occasionally been described, but the original three still seem to be the most important ones (Weimer, 1993b).

Fibrobacter succinogenes

Because the rod-shaped cellulolytic isolate produced succinate and was pleomorphic, Hungate (1950) named it *B. succinogenes*. However, *B. succinogenes* is not closely related to colonic bacteroides, and its 16S rRNA gene sequence indicated that it should be placed in a new genus, *Fibrobacter* (Montgomery *et al.*, 1988). *Fibrobacter* is not closely related to most other eubacteria and has been classified into a separate phylum (*Fibrobacteres*) containing no other genera (Gupta, 2004). *Fibrobacter* strains isolated from the rumen are typically called *succinogenes,* but there are two recognized species (Qi *et al.*, 2004). The 16S rRNA gene similarity of *F. succinogenes* S85 and *Fibrobacter intestinalis* NR9 is $< 93\%$, and the DNA homology is only 20% (Lin & Stahl, 1995; Béra-Maillet *et al.*, 2004). *Fibrobacter intestinalis* (Montgomery *et al.*, 1988) was originally isolated from the large intestine of a cow, but habitat distinction is not a clear-cut indicator of taxonomy at the species level.

Most studies with *F. succinogenes* have used strain S85 (Bryant *et al.*, 1959), and Halliwell & Bryant (1968) concluded that S85 was the most active cellulolytic bacterium isolated from the rumen. However, *F. succinogenes* binds tightly to feed and is not easy to isolate (Stewart *et al.*, 1981). The question then arises, as to how good a model S85 is. Recently, Béra-Maillet *et al.* (2004) compared freshly isolated strains with S85 and all but one were similar. Based on cellular and extracellular activity patterns, xylanase activities and ability to digest microcrystalline cellulose, they concluded that S85 is a 'good model for studying the fibrolytic properties of the species . . ..'

Ruminococcus albus and *R. flavefaciens*

The cellulolytic cocci (Hungate, 1950) were originally differentiated by pigmentation. The nonpigmented (white) isolates were called *R. albus* and the yellow-orange isolates were named *R. flavefaciens*, but pigmentation varies substantially. *Ruminococcus albus* 7 and several other strains have a distinct lemon-yellow color and, interestingly, these strains degrade cellulose considerably better than the white strain, *R. albus* 8. In a pure culture, *R. albus* produces ethanol, acetate, formate and hydrogen, while *R. flavefaciens* produces succinate instead of ethanol. 16S rRNA gene sequence analysis places the ruminococci within the Gram-positive bacterial phylum *Firmicutes* (Odenyo *et al.*, 1994a), but they do not have a typical Gram-staining reaction. *Ruminococcus albus* cells often stain Gram-negative and *R. flavefaciens* is Gram variable (Hungate, 1966). Most ruminococci cannot grow on pentose monosaccharides; however, many can utilize hemicelluloses as a source of energy, suggesting an ability to grow on oligomeric products of enzymatic hydrolysis (Dehority, 1973).

Culture-based vs. culture-independent enumeration

In the early 1950s, Marvin Bryant and his colleagues used the anaerobic techniques developed by Hungate to examine and enumerate cellulolytic ruminal bacteria from cattle fed different rations (Bryant & Burkey, 1953a, b; Bryant & Doetsch, 1954). Their results indicated that cellulolytic bacteria similar to those previously described by Hungate (1950) accounted for 5–28% of the nonadherent viable cell count. The viable cell number is typically only 10% of the total nonadherent cell number (Bryant & Burkey, 1953b; Hungate, 1966). The question then arises as to, does the rumen contain a large population of 'dead' bacteria or are microbiologists simply not able to culture the most numerous and perhaps most important ruminal bacteria? With the development of culture-independent, molecular-based techniques of bacterial enumeration, this has become a central question in virtually all fields of microbial ecology (Hugenholtz *et al.*, 1998). As noted by Amann *et al.* (1995), 'The frequent discrepancy between direct microscopic counts and numbers of culturable bacteria from environmental samples is just one of several indications that we currently know only a minor part of the diversity of microorganisms in nature.'

A further complication to the enumeration of cellulolytic bacteria is attachment and the inability of microbiologists to account for adherent populations. ATP determinations indicated that *c.* 70% of all rumen bacteria are firmly attached to feed particles and not easily removed (Forsberg & Lam, 1977). Because even small particles containing many individuals would only produce a single colony on a Petri plate or in an agar roll tube, significant underestimation is likely. With the advent of cultivation-independent molecular techniques, it was hoped that this bias could finally be overcome.

Using taxon-specific primers and appropriate standards that correct for species-specific variations in the efficiencies of the PCR, it is possible to estimate the fraction of the target gene in the bacterial population that is contributed by a particular taxon (Stevenson & Weimer, 2007). Because 16S rRNA gene copy number varies from one to 15 per genome among bacterial species and the average copy number in a particular rumen sample cannot be determined, interpreting the relative population sizes among bacterial species requires caution. Fortunately, the rRNA gene copy number for the three primary cellulolytic species has been reported to be three per genome (Frey *et al.*, 2006), and so the relative population sizes of these species can be compared directly.

However, even probes or PCR primers based on 16S rRNA genes are typically derived from cultivated bacteria, and the possibility that their specificity prevents detection of all members of a phylogenetically related group is difficult to exclude. This is illustrated by recent sensitive and reliable real-time PCR experiments (Stevenson & Weimer, 2007; Weimer *et al.*, 2008). Results indicated that *R. flavefaciens* was generally much more abundant than *R. albus,* but neither species accounted for $>1\%$ of the total 16S rRNA gene. However, when a genus-level primer for *Ruminococcus* was used, this value was 8%. These results indicate that not all ruminococci belong to the two known ruminal species. *Fibrobacter succinogenes* only represented *c.* 1% of the prokaryotic 16S rRNA gene copy number, but this value was based on a species- rather than a genus-level primer (Stevenson & Weimer, 2007).

Molecular-based, culture-independent enumeration methods have also permitted *in vitro* studies to elucidate interactions among cellulolytic species. Both batch (Odenyo *et al.*, 1994a, b) and continuous (Chen & Weimer, 2001) culture studies indicate that ruminal cellulolytic species compete intensely for both cellulose and cellobiose. The outcome of the interactions among the cellulolytic species appears to be based on a number of factors, including the rate of adherence to cellulose (Koike *et al.*, 2003), affinity for cellodextrin products of cellulose hydrolysis (Shi & Weimer, 1996), production of bacteriocins (Chen *et al.*, 2004) and the presence of noncellulolytic bacteria (Chen & Weimer, 2001).

Do we have suitable model organisms?

Discrepancies among total counts, culture-based and non-culture-based enumeration techniques can be discouraging, but less pessimistic views are possible if one views cultivated microorganisms simply as models. Do the cultured organisms have properties and characteristics that are representative of the bacteria in the mixed population? Are the activities of cultured organisms sufficient to explain the ability of the mixed population to perform a specific function? In the case of ruminal cellulose digestion, the answers (at least for now) seem to be yes. The cellulolytic bacteria originally isolated by Hungate (1950) can use ammonia as their sole source of nitrogen, have little capacity to take up and use amino nitrogen sources, require branched-chain volatile fatty acids to synthesize branched-chain amino acids and are sensitive to even modest decreases in pH, and all of these characteristics can influence cellulose digestion by mixed ruminal bacteria *in vitro* or *in vivo* (Bryant, 1973; Russell & Wilson, 1996; Atasoglu *et al.*, 2001).

The activities of the pure cultures to digest cellulose also seem to be adequate, as illustrated by the following calculation. The specific growth rates of cellulolytic ruminal bacteria on cellulose can be as high as $0.1\,h^{-1}$ (Wells & Russell, 1994; Maglione *et al.*, 1997), when observed yields are typically $0.2\,g\,cells\,g^{-1}$ cellulose (Shi & Weimer, 1992; Weimer, 1993a). Given these values, the specific activity of cellulose digestion can be $0.5\,g\,cellulose\,g^{-1}\,cells\,h^{-1}$. The amount of bacterial dry matter in the rumen is *c.* $10\,g\,L^{-1}$ (Hristov & Broderick, 1996), yielding a cellulose digestion rate of $5.0\,g\,cellulose\,L^{-1}\,h^{-1}$, which can be compared with cellulose that is actually digested in the rumen. If a cow with a 70-L rumen consumes 10 kg of forage dry matter per day, the cellulose concentration is $200\,g\,kg^{-1}$ dry matter, and if 50% of the cellulose is digested in the rumen, the cellulose digestion rate would be 1.0 kg cellulose per day or $0.60\,g\,cellulose\,L^{-1}\,h^{-1}$. This means that the cellulolytic population would need to be 0.6/5.0 or 12% of the bacterial population, a value within the range discussed above.

Mechanism of ruminal cellulose digestion

'True' cellulases cannot be isolated easily from cellulolytic ruminal bacteria. Cell-free extracts of ruminococci only solubilize a small amount of native cellulose, and extracellular culture fluid extracts from *F. succinogenes* S85 have virtually no activity, even though the cultures grow well on this substrate (Halliwell & Bryant, 1968; Béra-Maillet *et al.*, 2004). With the advent of the molecular era, rumen microbiologists had great hopes that the mechanism of ruminal cellulose degradation would finally be defined. The genome of *F. succinogenes* is now available, but our understanding of

ruminal cellulases and their action is still lacking (http://www.tigr.org/tdb/rumenomics/genomes.shtml).

Carboxymethylcellulose (CMC) hydrolysis is often used as a surrogate for native cellulose degradation, and a variety of CMCases have been heterologously expressed and characterized (Morrison *et al.*, 2003; Qi *et al.*, 2007). However, many (if not most) CMCases have little capacity to digest native insoluble cellulose and many noncellulolytic ruminal bacteria can hydrolyze CMC even though they cannot utilize native cellulose as a substrate for growth (Avgustin *et al.*, 1997; Fields *et al.*, 1998). Mixed ruminal bacteria from cattle fed hay had twice as much CMCase activity as bacteria from cattle fed 90% cereal grain, but CMCase activity was not strongly correlated with cellulose utilization (Fields *et al.*, 1998). Because all of the CMCase-positive, cellobiose-utilizing ruminal bacteria grew on β-glucan, CMCases seem to be a mechanism for utilizing water-soluble mixed β-glucans from cereal grains rather than native cellulose (Fields *et al.*, 1998).

The failure of crude extracts to digest native cellulose could be related to cellular organization. Some anaerobic bacteria that actively degrade native cellulose (e.g. *Clostridium thermocellum)* have organized structures called cellulosomes, in which numerous cellulases and other polysaccharide hydrolases are arranged on the cell surface in a manner thought to optimize plant cell wall hydrolysis (Bayer *et al.*, 2004). Microscopic examination suggests that ruminal fungi (Wood *et al.*, 1986) and *R. albus* (Pegden *et al.*, 1998) have similar structures, and this idea has been confirmed by biochemical and genomic studies. *Fibrobacter succinogenes* S85 attaches tightly to cellulose. However, there is no indication that it has cellulosomes, and the absence of known dockerin and cohesin sequences in its genome makes it extremely unlikely that it produces them (Park *et al.*, 2007). The ability of this species to degrade cellulose effectively instead appears to reside in its genomic encoding of an unusually large number (at least 33) of different cellulases and related enzymes (Qi *et al.*, 2007).

Another explanation for the inability of *F. succinogenes* S85 extracts to digest native cellulose may be feedback inhibition. Maglione *et al.* (1997) noted that the cellulose-dependent succinate production of *F. succinogenes* S85 cultures could be used to estimate first-order rates of native cellulose digestion, and results indicated that the cellulose digestion rate was closely correlated to the cellulose surface area. However, when thiocellobiose, a nonmetabolizable analog of cellobiose, was added, the rate of the cellulose-dependent succinate production decreased and Lineweaver–Burk plots indicated that thiocellobiose was a competitive inhibitor of cellulose digestion. The potential utility of end-product inhibition to *F. succinogenes* S85 is consistent with the effect of 'excess' cellobiose. N-limited, cellobiose-excess *F. succinogenes* S85 cell suspensions had little intracellular ATP or protonmotive force and viability declined dramatically (Maglione & Russell, 1997), but such declines were not observed when excess cellulose was added (Thomas & Russell, 2003).

Bacterial growth kinetics

Because ruminants depend on microbial protein as an amino acid source, the amount of bacterial mass produced in the rumen can often be as important to the animal as the amount of cellulose digested (Hungate, 1966). Microbiologists usually grow bacteria in batch cultures with soluble energy sources, under which conditions: $\mu x = -\,dS/dt \times Y$, where μ is the specific growth rate constant (h^{-1}), x is the bacterial cell mass, S is the substrate concentration and Y is the yield coefficient or the cell mass produced per unit of substrate (e.g. g cells g^{-1} glucose) (Ingraham *et al.*, 1983). Algebraic rearrangement indicates that $\mu = -\,dS/dt \times Y/x$. Thus, μ is proportional to $-\,dS/dt$. In continuous culture, Y is not a constant and, at low dilution rates, both μ and Y decline (Herbert *et al.*, 1956). The decrease in μ is due to a lower rate of S addition, but the decline in Y indicates another avenue of energy source utilization. Marr *et al.* (1963) called this nongrowth utilization 'maintenance' and, in their derivation, $-\,dS/dt \times Y = \mu x + ax$, where '$a$' is the specific maintenance coefficient (h^{-1}). This derivation has three assumptions. Firstly, 'a' is a constant. Secondly, Y is lower when $-\,dS/dt$ is lower because a greater fraction of the S is being diverted to maintenance. Thirdly, bacteria grow even faster if they have no maintenance requirement.

Pirt (1965) introduced a derivation that views maintenance differently, in which maintenance (m) is the amount of energy necessary to sustain a mass of bacteria for a specific time (g glucose g^{-1} bacteria h^{-1}). The two derivations are mathematically related: $m = a/Y_G$ or $m \times Y_G = a$, where Y_G is the theoretical maximum yield (without maintenance). Maintenance appears to entail at least three functions: (1) retention of ion gradients, (2) molecular turnover and (3) motility (Russell & Cook, 1995). *In vitro* studies indicate that ruminal cellulolytic bacterial have 'a' coefficients ranging from 0.01 to 0.02 h^{-1} g cellulose (g bacteria)$^{-1}$ h^{-1} (Shi & Weimer, 1992; Weimer, 1993a), which are as much as fourfold lower than those for noncellulolytic ruminal bacteria (Russell & Baldwin, 1979).

Stouthamer (1973) presented calculations based on standard pathways of biosynthesis and noted that Y_G would vary with the amount of ATP available as well as the cell composition. Y_G values for pure cultures of ruminal cellulolytics range from 0.23 to 0.30 g cells g^{-1} cellulose (Shi & Weimer, 1992; Weimer, 1993a). However, these estimates were based on assumed cell compositions and are as much as twofold lower than those of other ruminal bacteria (Russell & Baldwin, 1979). Estimation of Y_G is further complicated by changes in end products. For example, *R. albus* produces

ethanol *in vitro,* but this product is not formed *in vivo* (Iannotti *et al.*, 1973). *In vivo,* methanogens consume hydrogen and interspecies hydrogen transfer from *R. albus* allows it to make more acetate and increase ATP and cell production (Wolin & Miller, 1983).

When bacteria grow on soluble energy sources, the factor limiting $-dS/dt$ and μ is the capacity of the bacteria to take up substrate and the system is initially first order with respect to bacterial cells (rather than the substrate). However, the situation with insoluble cellulose in the rumen is different. The rumen always has a high concentration of cells and ruminal fluid inocula can be diluted five- to sixfold before there is a significant decrease in the cellulose digestion rate (Mouriño *et al.*, 2001). Based on these observations, the cellulose digestion rate ($-dS/dt$) in the rumen is primarily dictated by the surface area, physical properties and chemical nature of the cellulose and its surrounding matrix (Weimer *et al.*, 1990; Fields *et al.*, 2000; Kohn, 2003).

Ruminal cellulolytic bacteria do not secrete cell-free cellulases (Halliwell & Bryant, 1968) and only 'adherent' cells digest cellulose (Gong & Forsberg, 1989; Weimer, 1993a, b). However, cellulolytic and noncellulolytic ruminal bacteria can be cocultivated for long periods with cellulose as the sole energy source. Scheifinger & Wolin (1973) hypothesized that the noncellulolytics were using cellulose fragments released by the cellulases. However, more recent work indicates another avenue of cross-feeding (Wells *et al.*, 1995). Ruminal cellulolytic bacteria have intracellular phosphorylases (Alexander, 1961) and these reversible enzymes produce cellodextrins that can be secreted and made available to the nonadherent bacteria (Wells *et al.*, 1995).

Cellodextrin efflux is probably not an 'altruistic' characteristic of ruminal cellulolytic bacteria. When bacteria use phosphorylases to cleave hydrolytic bonds, hydrolases are not needed, one of the products is already phosphorylated (as glucose-1-phosphate) and alternative (ATP consuming) mechanisms of phosphorylation (e.g. glucose kinase) can at least be partially avoided. The equilibrium constant of cellobiose phosphorylase (determined in the nonruminal cellulolytic bacterium *C. thermocellum*) is 4 (Alexander, 1961). Experiments with *F. succinogenes* S85 indicated that the ratio of cellodextrin to cellodextrin with one more glucose unit ($n/n+1$) was 4 (Wells *et al.*, 1995) and the ratio of *F. succinogenes* S85 to the noncellulolytic ruminal bacterium, *Streptococcus bovis*, in cellulose cocultures was also 4 (Wells *et al.*, 1995). These results suggest that the bacterium receiving the cellodextrin receives approximately the same benefit as the one producing it and *vice versa*.

Fermentation vs. passage

Waldo *et al.* (1972) proposed that cellulose digestion in the rumen was a function of two competing, first-order rates (h^{-1}): the digestion rate (K_d) and the rate of passage of solids through the rumen (K_p). K_p is related to both the feed intake and the type of feed consumed (Allen & Mertens, 1988), but can be estimated from the passage of labeled feed particles (Satter, 1985). K_d is estimated from *in vitro* or *in situ* experiments and $K_d \times S = -dS/dt$ (Van Soest, 1973; Weimer *et al.*, 1990). Feeds for K_d determinations are finely ground to simulate the effects of rumination. If initial lag periods are ignored and one realizes that K_d sometimes declines after most of the cellulose is consumed, the formula $K_d/(K_d+K_p)$ provides a surprisingly good index of the amount of cellulose digested in the rumen (Fox *et al.*, 2004).

This model does not attempt to predict the passage of individual populations of bacteria from the rumen and assumes that bacteria produced in the rumen will pass from the rumen to the small intestine and that lysis and predation will eventually be subtracted. This constraint is due to the fact that methods of differentiating bacteria from each other and from feed particles are not yet accurate. For many years, the most commonly used microbial markers of bacterial were ^{15}N-labeled ammonia, the bacterial cell wall amino acid diaminopimelic acid (DAPA) and the 2-aminoethylphosphonic (AEP) of protozoa (Ling & Buttery, 1978). However, AEP is difficult to measure and the DAPA content varies widely among bacterial species. Moreover, DAPA present in cell wall material released due to intraruminal turnover of bacteria appears to persist much longer than the rapidly degraded proteins in the bacterial cytoplasm, a problem that would invalidate the use of DAPA as a microbial protein marker (Masson *et al.*, 1991). Nitrogen-15 measurements are labor intensive and require careful mathematical interpretation (Purser & Buechler, 1966). However, ^{15}N measurement is now considerably easier and less expensive and can be the method of choice for *in vivo* experimentation (G.A. Broderick, pers. commun.). In recent years, RNA determination has been simplified by measuring nonmetabolized urinary purine derivatives. Purine-derivative excretion provides an alternative to gut cannulae and more precise estimates of digesta flow from the rumen. However, as Broderick & Merchen (1992) noted 'no marker has proven completely satisfactory; hence, yield estimates are relative rather than absolute.'

Endogenous metabolism

Growth and maintenance can be described by $-dS/dt \times Y = \mu x + ax$, but this equation does not address 'starvation' (Dawes, 1985), when transmembrane gradients and ATP pools dissipate unless endogenous metabolism prevents this de-energetization. Bacteria with phosphotransferase systems (PTS) reserve a pool of phosphoenolpyruvate and can use it to reinitiate transport (Thompson & Thomas, 1977). However, cellulolytic ruminal bacteria lack a PTS

(Maas & Glass, 1991; Chow & Russell, 1992) and use stored glycogen to prevent cellular death (Wells & Russell, 1994). Because both maintenance and endogenous metabolism involve ion gradients, they appear, superficially, to be similar. However, *in vitro* and *in vivo* experiments with mixed ruminal bacteria indicate that the endogenous metabolism needed to preserve a subsequent rapid rate of cellulose degradation is 10-fold lower than the maintenance rate and they could starve for 24 h before the cellulose digestion rate decreased (Van Kessel & Russell, 1997).

Nitrogen, lysis and predation

Cellulolytic ruminal bacteria utilize ammonia as a nitrogen source and have little capacity to take up amino nitrogen (Bryant, 1973; Atasoglu *et al.*, 2001). ^{15}N labeling studies indicated turnover of microbial mass in the rumen that is typically explained by protozoal predation of bacteria (Nolan *et al.*, 1976; Firkins *et al.*, 1992). However, few protozoa ever leave the rumen (Weller & Pilgrim, 1974) and protozoa are very prone to lysis (Ankrah *et al.*, 1990). These observations implied that the converse was also possible, namely protozoal lysis, followed by bacterial metabolism (Wells & Russell, 1996a, b). *Prevotella* species may account for 40–60% of the bacterial 16S rRNA gene copy number *in vivo* (Stevenson & Weimer, 2007), but most have yet to be cultured and are probably not cellulolytic. Studies with other species of ruminal *Prevotella* indicate that lysis is not significant, but *Prevotella* species are subject to protozoal predation (Callaway & Russell, 2000). Some ruminal bacteria are very prone to lysis (Wells & Russell, 1996b). Bacteria must expand their cells while they are growing and use autolysins to cut the cell wall, to enable expansion (Koch, 1991). Ruminal bacteria have different types of autolytic regulation. *Fibrobacter succinogenes* uses a proteinase to degrade its autolysins once it reaches the stationary phase, and this degradation is triggered by energy source depletion (Wells *et al.*, 1995; Wells & Russell, 1996a, b; Maglione & Russell, 1997). If nitrogen or some other factor limits growth, the autolysins are not degraded and lysis occurs. Strains of *B. fibrisolvens* and *Ruminobacter amylophilus* lyse even faster than *F. succinogenes,* but little is known of their autolytic regulation. In *S. bovis*, autolysins are inactivated after cells reach the stationary phase. The inactivation of *S. bovis* autolysins is mediated by an unusual sugar residue (kojibiose) in its lipoteichoic acids. Kojibiose is a glucose disaccharide with a 1,2 linkage. If *S. bovis* is cultivated with 2-deoxyglucose, kojibiose is not produced, the autolysins are not inactivated and the cultures lyse (Bond *et al.*, 1999).

Ruminal acidosis

When cattle are fed an abundance of cereal grain along with fiber, volatile fatty acids can accumulate and ruminal pH can decline (Ash & Dobson, 1963). Ruminal pH is perhaps the most important environmental parameter affecting cellulose degradation by the ruminant animal (Mouriño *et al.*, 2001). Because ruminal cellulases with activity against native cellulose have not been extracted or purified, the effect of pH on the cellulases *per se* has not been directly determined. However, work with nonruminal bacteria indicated that most cellulases act by an acid catalysis mechanism and are activated (not inhibited) by mildly acidic pH (Russell & Wilson, 1996). The effect of pH on ruminal cellulose digestion is more easily explained by growth-related phenomena. None of the ruminal cellulolytic bacteria have evolved to grow at pH values significantly < 6.0 – a matter of some significance, as the rumens of both beef and dairy cattle under modern production conditions spend a substantial amount of the feeding cycle below this pH value.

Inhibition of growth by low pH is related to intracellular pH regulation (Russell & Wilson, 1996). Intracellular pH of acid-resistant fermentative bacteria declines when extracellular pH is low, but this strategy is only feasible if intracellular metabolism can withstand a decrease in pH (Russell & Diez-Gonzalez, 1998). Ruminal cellulolytic bacteria attempt to maintain a constant intracellular pH, but this leads to a large transmembrane pH gradient. Because undissociated volatile fatty acids can freely pass into the more alkaline interior, there is a logarithmic and toxic accumulation of intracellular volatile fatty acid anions (Russell & Diez-Gonzalez, 1998). Studies with *F. succinogenes* S85 demonstrated that creation of a larger pH gradient also leads to a nearly proportional decrease in the transmembrane electrical potential and cellobiose transport activity (Russell, 1987). Extracellular cellobiose (or possibly cellodextrins) then inhibits cellulases via end-product inhibition (Maglione *et al.*, 1997). Pitt *et al.* (1996) hypothesized that acidic ruminal pH would increase maintenance energy expenditures of cellulolytic ruminal bacteria, but this hypothesis was based on continuous culture data with *B. fibrisolvens* rather than the more active species of ruminal cellulolytic bacteria (Russell & Dombrowski, 1980). Because *F. succinogenes*, *R. albus* and *R. flavefaciens* did not exhibit a decline in cell yield before pH-dependent inhibition, there is little support for the idea that maintenance energy is affected (Russell & Dombrowski, 1980); in fact, estimates of m in cellulose-grown continuous cultures of both *F. succinogenes* (Weimer, 1993a) and *R. flavefaciens* (Shi & Weimer, 1992) decrease slightly with culture pH.

The potential impact of low pH and feedback inhibition on cellulose digestion in the rumen, however, must be coupled with the observation that cellulolytic ruminal bacteria can provide cellodextrins to noncellulolytic bacteria that are more resistant to acidic pH (Russell & Dombrowski, 1980; Russell, 1985; Mouriño *et al.*, 2001), helping to modulate the effect of pH on cellulose digestion and

allowing some cellulose digestion (albeit at a lower rate) at pH values < 6.0. Because even prolonged exposure of the ruminal cellulolytic bacteria to a low pH has little, if any, effect on their subsequent ability to digest cellulose (Hiltner & Dehority, 1983), ruminal pH only needs to remain > 6.0 (the minimum pH for growth of the cellulolytic bacteria) long enough to permit an average growth rate that exceeds their rate of passage (Mouriño *et al.*, 2001).

Only at pH values of 5.3 or lower is there a complete cessation of ruminal cellulose digestion, and values this low are usually not encountered in dairy cattle fed large amounts of cereal grain and modest amounts of forage. When the pH of the rumen is this low for an extended period, cellulolytic bacteria can no longer adhere to cellulose (Mouriño *et al.*, 2001) and the animal itself is severely affected by a variety of maladies (rumen ulcers, liver abscesses, founder and even death) (Nagaraja & Chengappa, 1998; Owens *et al.*, 1998).

A STELLA®-based model for ruminal cellulose digestion and bacterial growth

The question then arises: can our knowledge of ruminal cellulose digestion and bacterial growth be analyzed quantitatively? Many sophisticated modeling programs are now available. We chose to use a mature, commercial, dynamic simulation program STELLA® (Isee Systems Inc.; http://www.iseesystems.com), available for Microsoft Windows™ and Mac OS X™ platforms, and used to study a diversity of issues from economics to science. The STELLA® system has several advantages: (1) intuitive icon-based graphical interface, (2) stock and flow diagrams that provide an insight into how the system works and (3) causal loop diagrams that present relationships in a mechanistic fashion. The latest version can export models to the Internet for use with a web browser. STELLA® provides the biologist with a platform for translating mechanistic phenomena into quantitative models, without the necessity of prior modeling experience.

Richmond (2004) stressed the importance of excluding unnecessary details in any meaningful modeling effort. In keeping with this strategy, we focused on the major aspects of ruminal cellulose degradation: (1) ingestion, digestion and passage of feed particles, (2) maintenance and growth of cellulolytic bacteria and (3) pH effects. The model was based on assumptions that the rumen is a first-order system with respect to substrate (zero order with respect to cells) (Kohn, 2003) and that the amount of cellulose digested is a product of the digestion rate and passage rate (Waldo *et al.*, 1972). The model does not address bacterial interactions, differentiate among cellulolytic species or specifically account for adherent vs. nonadherent cells. However, the first-order rate of cellulose digestion is driven by the amount of cellulose remaining. As the cellulose concentration decreases and bacterial mass increases, an increasingly large fraction of the bacteria becomes nonadherent. Because the ruminal residence time of nonadherent bacteria is typically 24 h or less (Hungate, 1966), endogenous metabolism was not included. Protozoa and fungi were not included, based on observations that bacteria are usually much more important. The model cannot yet address potential cross-feeding between cellulolytic and noncellulolytic species, which could be important if the growth characteristics (e.g. Y_G and a) differed significantly between these two groups. Furthermore, the model does not address the passage of either group from the rumen, which will be addressed as the model is expanded to consider other aspects of rumen activity.

N-limitation was ignored because ruminants are surprisingly good at recycling urea to the rumen, where it is converted to ammonia, and ruminal ammonia can be inexpensively derived from dietary nonprotein nitrogen (Nolan & Dobos, 2005). Lysis and predation play significant roles in ruminal ecology, but the impact on cellulose digestion is not yet clear. Two questions immediately arise. Are cellulolytics more prone to lysis than other ruminal bacteria? Are protozoa able to engulf attached as well as nonadherent bacteria? Until these questions can be answered, lysis and predation were considered beyond the scope of this model, but could easily be incorporated (see Fig. 1).

Figure 1 is a STELLA® schematic that shows ruminal cellulose digestion, and the generated equations are shown in Table 1. A key advantage of STELLA® is the ability to evaluate the model graphically during construction. Alternatively, individual pieces can be segregated by multiplying a 'down- or upstream' flux by zero. Initially, cellulose was evaluated with a K_d of $0.1\,h^{-1}$. K_d values can be as high as $0.1\,h^{-1}$, but values as low as $0.03\,h^{-1}$ have also been reported (Van Soest, 1973; Lechtenberg *et al.*, 1974). K_p values range from 0.03 to $0.08\,h^{-1}$ (Satter, 1985) and the model accommodates variations in K_p as well as K_d. Cellulose utilization based on a K_d of $0.1\,h^{-1}$ and a K_p of $0.05\,h^{-1}$ is shown in Fig. 2a. Because K_d was twice as high as K_p, twice as much cellulose was digested, as passed. Figure 2b shows the effect of varying K_d with K_p set at $0.05\,h^{-1}$, but an alternative strategy of varying K_p with a set K_d could easily be used.

'Digested cellulose' provides: (1) ATP for maintenance, (2) ATP for growth and (3) carbon to synthesize cells. Fluxes 'ATP for maintaining' and 'Growing' were initially based on μ and a, but became rate-limiting steps, and there was a large but transient buildup of 'Digested Cellulose.' Because cellobiose and cellodextrins never accumulate in the rumen, another strategy was needed. The strategy chosen was to 'partition' the 'Digested Cellulose' based on the relative values of K_d, μ and a by introducing a converter, 'μ vs. a,' that was computed as $(K_d - a)/a$ (Fig. 1). This converter was only multiplied by 'Growing,' and 'ATP for Maintaining' was left unaffected. Because neither 'Growing' nor 'ATP for

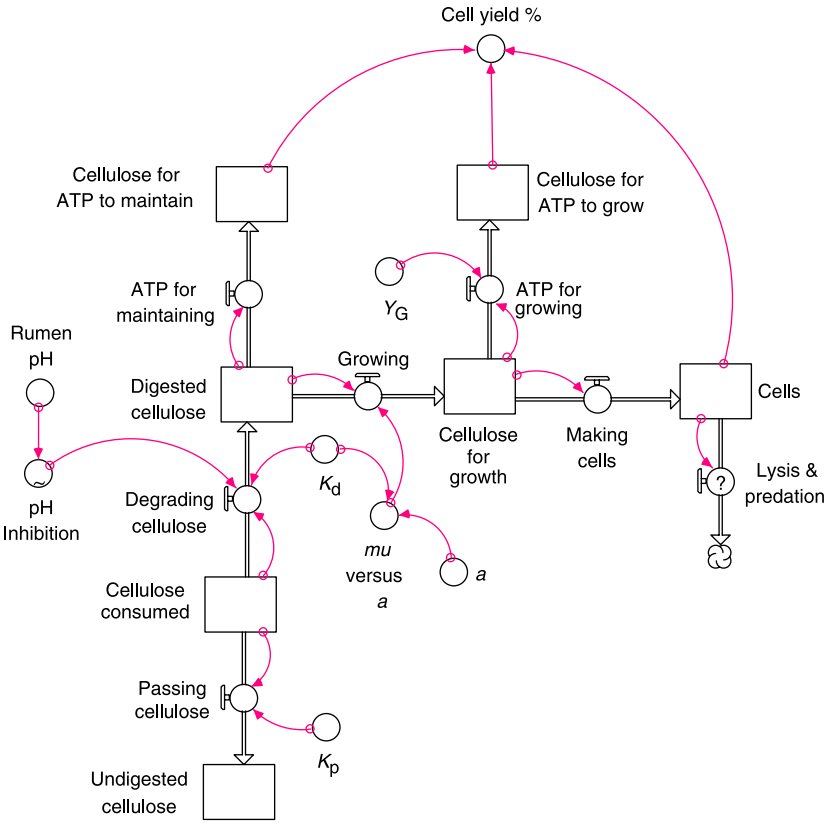

Fig. 1. A schematic showing the STELLA® program that was developed to relate ruminal cellulose digestion with the rate of cellulolytic bacteria.

Table 1. Equations of STELLA to model cellulose digestion

Cells(t) = Cells($t - dt$)+(Making_Cells − Lysis_&_Predation) × dt
  INITIAL: Cells = 1
  INFLOWS:
    Making_Cells = Cellulose__for_Growth
  OUTFLOWS:
    Lysis_&_Predation = unspecified for now
Cellulose_Consumed(t) = Cellulose_Consumed($t - dt$)+
(− Degrading_Cellulose − Passing_Cellulose) × dt
  INITIAL: Cellulose_Consumed = 10 000
  OUTFLOWS:
    Degrading_Cellulose = Cellulose_Consumed × K_d × pH_Inhibition
    Passing_Cellulose = Cellulose_Consumed × K_p
Cellulose_for_ATP_to_Maintain(t) = Cellulose_for_ATP_to_
Maintain($t - dt$)+(ATP_for_Maintaining) × dt
  INITIAL: Cellulose_for_ATP_to_Maintain = 0
  INFLOWS:
    ATP_for_Maintaining = Digested_Cellulose
Cellulose_for__ATP_to_Grow(t) = Cellulose_for__ATP_to_
Grow($t - dt$)+(ATP_for__Growing) × dt
  INITIAL: Cellulose_for__ATP_to_Grow = 0
  INFLOWS:
    ATP_for__Growing = Cellulose__for_Growth × ((1/Y_G) − 1)
Cellulose__for_Growth(t) = Cellulose__for_Growth($t - dt$)+
(Growing − ATP_for__Growing − Making_Cells) × dt
  INITIAL: Cellulose__for_Growth = 0
  INFLOWS:
    Growing = Digested_Cellulose × μ__vs.__a

Table 1. Continued.

  OUTFLOWS:
    ATP_for__Growing = Cellulose__for_Growth × ((1/Y_G) − 1)
    Making_Cells = Cellulose__for_Growth
Digested_Cellulose(t) = Digested_Cellulose($t - dt$)+(Degrading_
Cellulose − ATP_for_Maintaining − Growing) × dt
  INITIAL: Digested_Cellulose = 0
  INFLOWS:
    Degrading_Cellulose = Cellulose_Consumed × K_d × pH_Inhibition
  OUTFLOWS:
    ATP_for_Maintaining = Digested_Cellulose
    Growing = Digested_Cellulose × μ__vs.__a
Undigested_Cellulose(t) = Undigested_Cellulose($t - dt$)+(Passing_
Cellulose) × dt
  INITIAL: Undigested_Cellulose = 0
  INFLOWS:
    Passing_Cellulose = Cellulose_Consumed × K_p

a = 0.02.
Cell_Yield_% = Cells/(Cells+Cellulose_for_ATP_to_Maintain+Cellulose_
for__ATP_to_Grow) × 100.
K_d = 0.06.
K_p = 0.05.
μ__vs.__a = ($K_d - a$)/a.
Rumen__pH = 7.
Y_G = 0.2.
pH_Inhibition = GRAPH(Rumen__pH).
(5.00, 0.00), (5.20, 0.1), (5.40, 0.25), (5.60, 0.4), (5.80, 0.55), (6.00, 0.7), (6.20, 0.85), (6.40, 0.995), (6.60, 1.00), (6.80, 1.00), (7.00, 1.00).

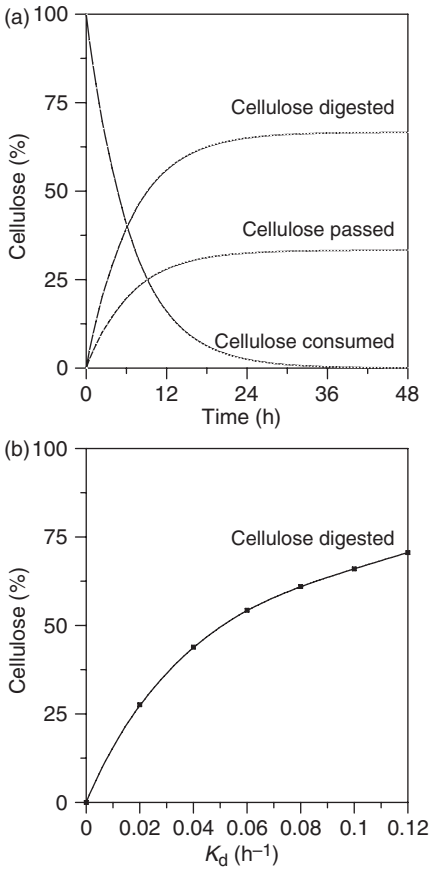

Fig. 2. (a) Amount of cellulose (%) that was consumed, digested in the rumen or passed from the rumen undigested. In this particular example the digestion rate (K_d) was 0.1 h^{-1} and the passage rate (K_p) was 0.05 h^{-1}. (b) Cellulose consumption when K_d was varied from 0 to 0.2 h^{-1} and K_p was set at 0.05 h^{-1}.

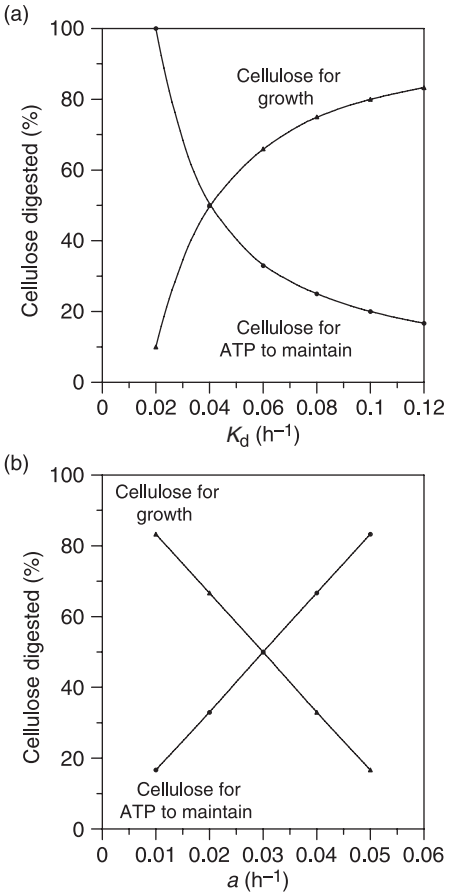

Fig. 3. Impact of digestion rate, K_d (a) or the maintenance coefficient, a (b), on the utilization of 'Digested Cellulose' for either growth (*Cellulose for Growth*) or maintenance (*Cellulose for ATP to Maintain*).

Maintaining' was rate limiting, 'Digested Cellulose' did not accumulate. The effect of K_d (when $a = 0.02\ h^{-1}$) on 'Cellulose for ATP to Maintain' vs. 'Cellulose for Growth' is shown in Fig. 3a. Sensitivity analysis of a with constant K_d is shown in Fig. 3b. Based on these results, both of these physiological characteristics are important.

'Cellulose for Growth' was then partitioned into cellulose used to make 'Cells' and 'Cellulose for ATP to Grow' (Fig. 1), based on the idea that 'Y_G' could also be a variable, and another converter (Y_G) adjusts 'ATP for Growing.' The effect of changes in Y_G is shown in Fig. 4. As mentioned above, declines in ruminal pH can inhibit cellulose digestion, and this is accommodated by a graphical converter for 'Degrading Cellulose' (Fig. 5). This graph was derived from a regression equation of experiments conducted with mixed ruminal bacteria *in vitro* (Mouriño *et al.*, 2001). The effect of pH on 'Digested Cellulose' is shown in Fig. 6. The decision to have pH affect K_d rather than Y_g, μ or a was based on the observation that acidic pH increases interconversion of the

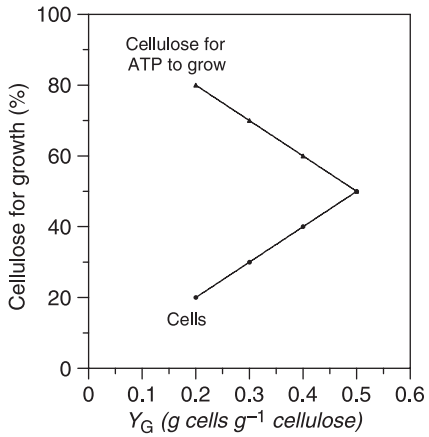

Fig. 4. Impact of theoretical maximum growth yield (Y_G) on the utilization of 'Cellulose for Growth' for either ATP production needed to make cells (*Cellulose for ATP to Grow*) or actually making cell material (*Cells*).

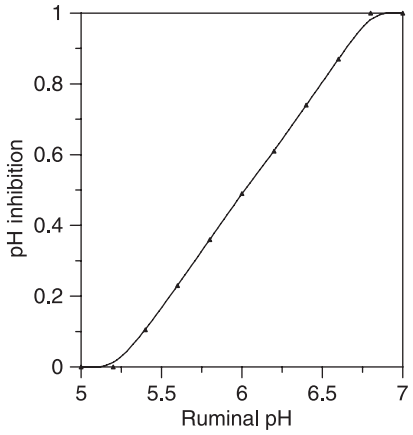

Fig. 5. Inhibition of cellulose digestion (K_d) by ruminal pH. When the value is less than 1.0, K_d is decreased proportionally.

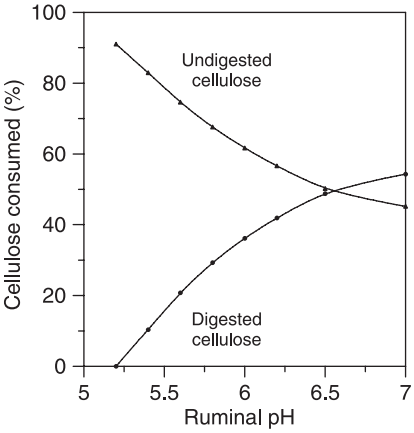

Fig. 6. Effect of ruminal pH on 'Cellulose Digested' and the passage of 'Undigested Cellulose' from the rumen. The digestion rate, K_d, and passage rate, K_p, were 0.06 and 0.05 h^{-1}, respectively.

transmembrane pH and electrical gradients, anion accumulation, loss in transport activity (Russell, 1987) and subsequent end-product inhibition of cellulases (Maglione *et al.*, 1997) (see 'Ruminal acidosis'). Because the data in Fig. 5 were derived from studies with mixed cultures of ruminal bacteria rather than pure cultures, the 'modulation' caused by cellodextrin cross-feeding is already accounted for.

As mentioned previously, the efficiency of bacterial growth in the rumen can significantly impact the availability of amino acids to the animal, and bacterial growth is influenced by a variety of factors that can act in an additive fashion (Fig. 1). By dividing 'Cells' by the sum of 'Cellulose for ATP,' 'Cellulose for ATP to Grow' and 'Cells,' it is possible to calculate 'Cell Yield %' as well. Figure 7 shows that variations in Y_g and K_d can have a large impact on cell yield when $a = 0.02\ h^{-1}$. However, it should be noted that at very low K_d values, cellulose digestion is virtually independent of Y_G.

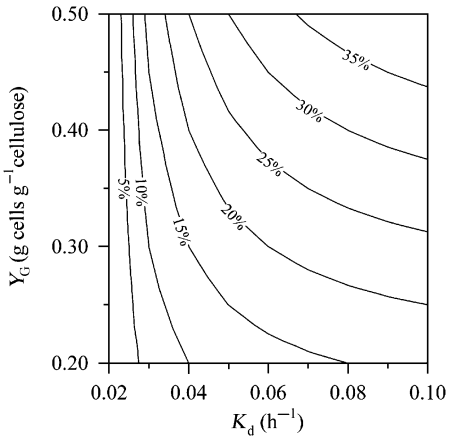

Fig. 7. Effect of digestion rate (K_d) and theoretical maximum growth yield (Y_G) on the cell yield of cellulolytic ruminal bacteria. Values on each contour are expressed as % of g cells g^{-1} cellulose.

Limitations of the model and outlook for future research

Richmond's (2004) instructions for constructing and evaluating a model are very straightforward: 'despite the fact that all models are wrong, *you have no choice but to use them* – no choice that is, if you are going to *think*.' In keeping with this instruction, some limitations of the model are described. The digestion vs. passage relationship originally proposed by Waldo *et al.* (1972) was based on a steady-state model of ruminal fermentation, but animals often consume feed in a discontinuous rather than a continuous fashion. Another simplification is the idea that feed particles are in a form that can immediately be digested or leave the rumen. When animals consume large feed materials, rumination is needed to grind them to a size that can pass through the omasum to the lower gut. However, because this process would confer a time delay for both K_p and K_d, there is at least some compensation. The model in its simplest form only uses a single form of cellulose. However, cellulose can be derived from more than one feed source, and K_d can vary. One method of handling this latter complexity is to use weighted averages that adjust K_d in proportion to the amount and type of feeds ingested. Probably the greatest limitation of the model involves pH. If the rumen were truly operating at a steady state, pH could be set at a constant value. However, diets that promote ruminal acidosis contain large amounts of cereal and are typically consumed in meals rather than continuously. All of these considerations raise yet another question: should the model be used to address a single animal or a large group of animals consuming a similar diet? Because animals differ greatly in their feeding behavior, limitations would be greater for a single animal rather than the average of a group. Another obvious reservation revolves around the importance of a and Y_G in determining the

magnitude of cell production, and the scarcity of data in this area of research. Because sensitivity analyses (Figs 3, 4 and 7) demonstrate that these parameters are important determinants of cell production from cellulose, additional research is warranted.

Future additions to the STELLA® model will include other aspects of ruminal fermentation that can affect animal productivity, including other major end-products of rumen fermentation: fermentation acids (the primary energy sources for the animal), CO_2, CH_4 and excreted nitrogen. By better balancing the rate of carbohydrate and protein degradation in the rumen, it may be possible to use STELLA® to improve the efficiency of nitrogen use in the ruminants.

Ruminal vs. industrial fermentations of plant biomass

Nearly 60 years ago, Robert Hungate (1950) compared ruminal and industrial fermentations and concluded that the rumen was superior. 'In summary, an industrial cellulose fermentation might be profitable if the cost of collection of raw materials could be minimized through the use of numerous small plants, if the small plants could be cheaply constructed, if the operation could be made automatic to decrease necessary personnel, and if the concentration of cellulose fermented could be increased by continuous removal of fermentation products. Although such a situation is at present quite out of the question as an industrial process, it is almost an exact specification of the ruminant animal, a small fermentation unit which gathers the raw materials, transfers it to the fermentation chamber, and regulates its further passage, continuously absorbs the fermentation products and transforms them into a few valuable substances, like meat, milk, etc. To these advantages must be added the crowning adaptation: the unit replicates itself.'

The United States has initiated a massive program to convert corn into ethanol to partially replace petroleum with a 'biofuel' (Groom *et al.*, 2008). However, most people agree that utilization of starch is only a temporary solution and that the technology will eventually utilize cellulosic materials (e.g. switchgrass) (Searchinger *et al.*, 2008). Conversion of cellulose to ethanol is constrained by the fact that the most commonly exploited ethanol-producing microorganisms, the yeasts, lack cellulases. Some people have advocated the use of fungal cellulases in combination with traditional yeast fermentation. However, despite years of intensive research, this process is not yet economical (Himmel *et al.*, 2007). Others have proposed use of the fermentative anaerobe, *C. thermocellum* (Lu *et al.*, 2006), in a single-step process 'consolidated bioprocessing' (Lynd *et al.*, 2002), but this cellulose-degrading bacterium prefers to produce end products that yield more ATP than ethanol (e.g. acetate), and it has a significantly lower tolerance of ethanol than yeast (Demain *et al.*, 2005). Currently, industrial fermentations do not have economical systems of continuous end-product removal, which is a major shortcoming.

Hungate's (1950) comparison was eloquent, but did not directly address the property most apt to limit either ruminal or industrial fermentation of cellulose, namely available surface area. The ruminant solves this problem by selecting forages that (1) are not highly lignified, (2) have thin cell walls and (3) can be ground (ruminated) into very small particles so that the cellulases can have a greater access (Jung *et al.*, 2004). Industrial cellulose fermentations could, in theory, also use mechanical procedures to reduce the particle size, but chemicals coupled with nonambient temperatures or pressures appear to be more practical. The most commonly used chemicals are either strong acids that separate the hemicellulose and lignin from the cellulose or alkali (NaOH or NH_3) that dissolves the lignin. The economics of these treatments are still questionable.

Conclusions

The rumen is the best-understood cellulose-digesting ecosystem in nature and it appears that previously isolated and characterized cellulolytic bacteria provide a reasonable model of ruminal cellulose digestion. Using STELLA® software, we have devised a mathematical model that addresses all of the major aspects of ruminal cellulose degradation: (1) ingestion, digestion and passage of feed particles, (2) maintenance and growth of cellulolytic bacteria and (3) pH effects. Simulations indicated that all of these parameters were potentially important determinant of cellulose digestion and cell production in the rumen. Continuously operated industrial cellulose fermentations that are as robust as the rumen have not yet been developed. However, our STELLA® model could be modified to accommodate an industrial batch culture that is eventually limited by the end product (ethanol).

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